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"Inhibitors of the leukocyte proMMP9/beta2 integrin complex"
(Leukosyytti-proMMP-9/beta2-integriinikompleksin inhibiittorit)

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Inhibitors of the leukocyte proMMP9/β_2 integrin complex

INTRODUCTION

The leukocyte integrin family consists of four heterodimeric glycoproteins with specific α–chains (α_L, α_M, α_X, or α_D) and a common β_2-chain (CD18). They play an essential role in mediating adhesion of cells in the immune system (1). The major ligand-binding site locates to an 200 amino acid long sequence within the α–chain called I or inserted domain, which is homologous to the A domains of von Willebrand factor, repeats of cartilage matrix protein and collagen (2).

Among the β_2 integrins, $\alpha_M\beta_2$ is the most promiscuous binder being able to interact with a multitude of unrelated ligands. These include ICAM 1 to 5, complement fragment iC3b, fibrinogen, uPAR, E-selectin and various extracellular matrix proteins (see (3) and references therein). The integrin has also been shown to have a capacity to bind certain enzymes, but whether this is important for leukocyte adhesion or immune reactions is unclear. Such enzymes showing integrin-binding activity are catalase (4), myeloperoxidase (5) and the proteinases elastase (6) and urokinase (7).

Extensive work has been done to identify the ligand binding sites in β_2 integrin I domains, but less is known about the interacting ligand regions (8,9). Recently, the structure of an α_L I domain/ICAM-1 complex was reported (10). Low molecular weight peptides binding to the β_2 integrins are useful reagents to study integrin function, and such peptides have been derived from ICAM-2 (11), fibrinogen (12), and Cyr61 (13). We have used phage display libraries to study the peptide binding specificity of integrins and to develop potential drug leads. In our previous study, we isolated the biscyclic peptide CPCFLLGCC (LLG-C4)[1] as the most active binder to the purified $\alpha_M\beta_2$ integrin (14). Leukocytes can efficiently adhere to the immobilized LLG-C4 peptide via the $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins.

We have now extended phage display screenings to the purified α_M I domain. This has resulted in the identification of a novel I domain-binding tetrapeptide motif D/E-

D/E-G/L-W, which is found on some of the known β_2 integrin ligands and interestingly also on the catalytic domain of MMPs. We show that the D/E-D/E-G/L-W motif mediates binding between an MMP and β_2 integrin, and proMMP-9 gelatinase, the major MMP of leukocytes (15-18), occurs in complex with the $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins in leukemic cell lines following cellular activation. The peptide inhibitors of the integrin-MMP complex prevent leukaemia cell migration, suggesting a role for the complex in cell motility.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

The antibodies MEM170 and LM2/1 were against the α_M and the MEM-83 and TS2/4 antibodies against the α_L integrin subunit (19,20). The monoclonal antibody 7E4 (21) reacted with the common β_2-chain of the leukocyte integrins. The α_M antibody OKM10 was obtained from the American Type Culture Collection, ATCC, Rockville, MD (22). A monoclonal antibody against ICAM-5 (TL3) (23) was used as an antibody control. The monoclonal anti-MMP-9 antibody (GE-213) and anti-MMP-2 antibody (Ab-3) were obtained from Lab Vision Corporation (Fremont CA) and from Oncogene™ research products, respectively. Affinity purified rabbit anti-MMP-9 polyclonal antibodies were from the Borregaard laboratory (24). The rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody was from Dakopatts a/s (Copenhagen, Denmark). Inh1 (2R-2-(4-biphenylylsulfonyl)amino-N-hydroxy propionamide) was purchased from Calbiochem, La Jolla, CA. Human recombinant ICAM-1 was obtained commercially by R&D systems (Minneapolis, MN). ICAM-1-Fc fusion protein was expressed in chinese hamster ovary cells and purified as described (14). The synthetic peptides CTT, STT, LLG-C4 and RGD-4C were obtained as previously described (14,25). W→A CTT was ordered from Neosystem, Strasbourg, France. ProMMP-2 and proMMP-9 were obtained commercially (Roche). In zymography, the commercial proMMP-9 showed the 92kDa monomer, 200 kDa homodimer, and 120 kDa NGAL complex bands. The integrins $\alpha_1\beta_1$ and $\alpha_3\beta_1$ were purchased from Chemicon International (Temecula, CA). Human plasma fibrinogen and lovastatin were from Calbiochem.

Phage display

Phage display selections were made using a pool of random peptides $CX_{7-10}C$ and X_{9-10}, where C is a cysteine and X is any amino acid (14,25). Briefly, α_M I domain-GST or GST fusion protein was immobilized on microtiter wells at 20 μg/ml concentrations and the wells were blocked with BSA. The phage library pool was first subtracted on wells coated with GST and then unbound phage was transferred to α_M I domain-GST-coated wells in 50 mM Hepes/5 mM $CaCl_2$/1 μM $ZnCl_2$/150 mM NaCl/2% BSA (pH 7.5). After three rounds of subtraction and selection, individual phage clones were tested for binding specificity and the sequences of the phage that specifically bound to the I domain were determined (14).

Peptide biosynthesis and chemical synthesis

The phage peptides were initially prepared biosynthetically as intein fusions. The DNA sequences encoding the peptides were PCR cloned from 1 μl aliquots of the phage-containing bacterial colonies that were stored at -20 C. The forward primer was 5'-CCTTTCTGCTCTTCCAACGCCGACGGGGCT-3' and the reverse primer 5'-ACTTTCAACCTGCAGTTACCCAGCGGCCCC-3'. The PCR conditions included initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C 30 sec, 55°C 30 sec, and 72°C 30 sec. The PCR products were purified using QIAGEN Nucleotide removal kit. They were then digested with SapI and PstI restriction enzymes and ligated to a similarly digested and phosphatase treated pTWIN vector (New England Biolabs). Correct insertions were verified by DNA sequencing. Intein fusion proteins were produced in *E. coli* strain ER2566 and affinity purified on a chitin column essentially as described (26). The peptide was cleaved on the column, eluted and finally purified by HPLC. Chemical peptide synthesis was done using Fmoc-chemistry as described and the sequences were verified by mass spectroscopy (26).

Phage binding assay

Phage (10^8 infective particles/well) in 50 mM Hepes/5 mM $CaCl_2$/1 μM $ZnCl_2$/0.5% BSA (pH 7.5) were added to microtiter wells coated with I domain-GST fusion or GST (20 ng/well). The phages were allowed to bind in the absence or presence of a competitor peptide (15 μM) for one hour followed by washings with PBS containing

0.05% Tween 20. The bound phage was detected using 1:3000 dilution of a peroxidase-labelled monoclonal anti-phage antibody (Amersham Biosciences) and o-phenylenediamine dihydrochloride as a substrate. The reactions were stopped by addition of 10% H_2SO_4 and the absorbance was read at 492 nm using a microplate reader.

Pepspot

The peptides were synthesized on cellulose membranes as described (27). The membrane was blocked with 3% BSA in TBS containing 0.05% Tween 20, and incubated with 0.5-5 µg/ml α_M I domain for 2 h at room temperature. The DDGW peptide was used as a competitor at a 50 µM concentration. Bound α_M I domain was detected using the monoclonal antibody LM2/1 (1 µg/ml) or MEM-170 (5 µg/ml) and peroxidase-conjugated rabbit anti-mouse antibody (1:5000 dilution) followed by chemiluminescence detection.

Cell culture

The human HT1080 fibrosarcoma and THP-1 and Jurkat leukemic lines were obtained from ATCC and maintained as described previously (20, 25, 28). OCI/AML-3, derived from the primary blasts of an AML patient (29) was maintained in 10% FBS/RPMI supplemented with L-glutamine, penicillin and streptomycin. Cell viability was assessed with a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay according to the instructions of the manufacturer (Roche).

Purification of integrins

$\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1) and $\alpha_X\beta_2$ (CD11c/CD18) integrins were purified from human blood buffy coat cell lysates by adsorption to the anti-CD11a (TS 2/4), anti-CD11b (MEM170), or anti-CD11c (3.9) antibodies linked to protein A-Sepharose CL 4B. The integrins were eluted at pH 11.5 in the presence of 2 mM $MgCl_2$, and 1% n-octyl glucoside as described previously (28).

Expression and purification of GST fusion proteins

The α_L, α_M, and α_X I-domains were produced as GST fusion proteins in *E. coli* strains BL 21 or JM109 and purified by affinity chromatography on glutathione-coupled

beads (30, 31). GST containing CTT in the C-terminus was constructed using the protocols described for LLG-C4-GST (14) and glutathione-coupled beads were employed for purification. The purity of the GST-fusion proteins was confirmed by SDS-PAGE with Coomassie Blue staining and Western blot analysis. For pepspot analysis, GST was cleaved from the α_M I domain with thrombin.

Binding of MMPs to purified integrins

The purified I domains (GST-α_M, GST-α_L, GST-α_X), or integrins ($\alpha_M\beta_2$, $\alpha_L\beta_2$, $\alpha_X\beta_2$, $\alpha_1\beta_1$) (1 µg/well) were immobilized in 20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$, pH 7.4. The wells were washed with PBST (10 mM phosphate, 140 mM NaCl, pH 7.4, containing 0.05% Tween20) and blocked with 3% BSA in PBST. ProMMP-2, proMMP-9, or the p-aminophenyl mercuric acetate (APMA) or trypsin-activated forms (32) were incubated for 2 h at room temperature. In the inhibition experiments, CTT and Inh1 were first preincubated with the proMMPs for 30 minutes at room temperature. The wells were washed three times and incubated with anti-MMP-9 (GE-213) or anti-MMP-2 (Ab-3) antibody at a 2 µg/ml concentration in PBST for 1 h. Bound antibodies were detected using peroxidase-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) and o-phenylenediamine dihydrochloride as a substrate.

Coprecipitation of β_2 integrin and progelatinases

Serum-free conditioned medium containing proMMP-2 and proMMP-9 was collected from human HT-1080 fibrosarcoma cells grown in the presence of 100 nM phorbol ester 4β-Phorbol 12,13-dibutyrate (PDBu) (Sigma-Aldrich, St. Louis, MO) overnight at +37°C. A 500 µl volume of the supernatant was incubated with 100 ng of GST-α_M, GST-α_L, or GST-α_X I-domain or $\alpha_M\beta_2$ integrin for 3 h at 25°C. GST and GST-LLG-C4 were used to determine non-specific binding. CTT, STT, LLG-C4, and ICAM-1 were used as competitors at a 200 µg/ml concentration, and the antibodies LM2/1 and TL3 at 40 µg/ml. After an hour incubation at +4°C, complexes of I-domain and gelatinases were pelleted with Glutathione Sepharose. Integrin complexes were captured by incubating first with the OKM10 antibody for 3 h at +4°C and then with protein G Sepharose for 1 h. After centrifugation and washing, samples were analyzed

by gelatin zymography on 8% SDS-polyacrylamide gels containing 0.2 % gelatin (32).

Effect of peptides on proMMP-9 release from cells

THP-1 cells (40 000/100 µl) were incubated in serum-free RPMI medium for 48 h in the absence or presence of 200 µM peptide as described in the text. Aliquots of the conditioned media were analyzed by gelatin zymography.

Interaction between CTT and proMMP-9

CTT-GST and GST control (5 µg/well) were coated overnight on 96-well microtiter plates in 50 µl TBS followed by blocking of the wells by BSA. proMMP-9 or APMA-activated form (80 ng/well) was incubated in the absence or presence of competitors for 2 h in 50 µM Hepes buffer containing 1 % BSA, 5 mM $CaCl_2$, and 1 µM $ZnCl_2$ (pH 7.5). After washing, bound MMP-9 was determined with anti-MMP-9 and HRP-conjugated anti-mouse IgG as described above. To examine complexing of CTT with proMMP-9 in cell culture, THP-1 cells were activated with PDBu for 30 min and then incubated with CTT, W→A CTT, or Inh1 (each 200 µM) at +37°C in serum-free medium. Samples were taken from the media at 0, 1, 2, 3, 4, and 5 h time points and analyzed by zymography and Western blotting with polyclonal anti-MMP-9 antibodies. Experiments with HT-1080 cells were performed similarly except that the medium samples were collected after 6 h.

Cell surface labelling, immunoprecipitation and immunoblotting

Non-activated or PDBu-activated THP-1 cells (1×10^7) were subjected to surface labelling using periodate tritiated sodium borohydride (33). The [^{3}H]-labelled cells were lysed with 1% (v/v) Triton X-100 in PBS, clarified by centrifugation and precleared with protein G-Sepharose. The lysate was immunoprecipitated with polyclonal anti-MMP-9, α_M (OKM-10) or β_2 (7E4) antibodies. After an hour incubation at +4°C together with protein G-Sepharose, immunocomplexes were pelleted, washed and resolved on 8-16% SDS-PAGE gels (Bio-Rad, Hercules CA). The gels were treated with an enhancer (Amplify, Amersham Biosciences), dried and exposed. Non-labelled THP-1 cells (1×10^7) were similarly lysed and immunoprecipitated as above. The samples were resolved on 4-15% SDS-PAGE gels

and transferred to nitrocellulose membranes. Immunodetection was performed with α_M (MEM170) antibody (10 μg/ml) followed by peroxidase-conjugated anti-mouse IgG and chemiluminescence detection (Amersham Biosciences). The membranes were stripped of bound antibodies and reprobed with monoclonal α_L chain (TS2/4) or polyclonal anti-MMP-9 antibodies.

Immunofluorescence

Immunofluorescence was performed on resting cells or the cells activated with PDBu for 30 min. A portion of the cells was treated with ICAM-1 or CTT to block β_2 integrins or gelatinases, respectively. Cells were bound to poly-L-Lysine coated cover slips, fixed with methanol for 10 min at –20°C or with 4% paraformaldehyde for 15 min at +4°C, and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min followed by several washings. The cover slips were incubated with rabbit anti-MMP-9 polyclonal and mouse anti-α_M (OKM-10) antibodies diluted 1:500. After washing with PBS, the secondary antibodies, rhodamine (TRITC)-conjugated porcine anti-rabbit or FITC-conjugated goat anti-mouse $(Fab')_2$ (Dakopatts a/s, Copenhagen, Denmark) were incubated at a 1:1000 dilution for 30 min at room temperature. The samples were mounted with moviol, incubated in the dark for 2 days, and examined by a confocal microscope (Leica multi band confocal imagine spectrophotometer) at a 400x magnification or a fluorescence microscope (Olympus Provis 70) at a 60x magnification.

Cell adhesion and migration

Fibrinogen and ICAM-1-Fc were coated at 40 μg/ml in TBS at +4°C. Peptides (2 μg/well) were coated in TBS containing 0.25% glutaraldehyde at +37°C. The wells were blocked with 1% BSA in PBS. THP-1 cells (50 000/well) with or without PDBu activation were added in 0.1% BSA-RPMI medium in the presence or absence of 200 μM peptides or monoclonal antibodies at 50 μg/ml. After 30-35 minutes the wells were washed with PBS to remove non-adherent cells and the adhesive cells were quantitated by a phosphatase assay. The cell migration assay was conducted using transwell migration chambers (8 μm pore size, Costar) in serum-containing medium as described (14). Briefly, the membranes were coated on the upper and lower surface with 40 μg/ml GST, LLG-C4-GST, or left uncoated. The wells were blocked with

10% serum-containing medium for 2 h. THP-1 cells (50 000/100 μl) or HT1080 (20 000/100 μl) were preincubated with the peptides for 1 h before transfer to the upper chamber. The lower chamber contained 500 μl of the medium without the peptides. The cells were allowed to migrate to the lower surface of the membrane for 16 h and then stained with crystal violet and counted.

RESULTS

Identification of the α_M I domain-binding peptide motif D/E-/D/E-G/L-W

Using phage peptide display libraries, we selected peptides that interact with the α_M I domain. GST-binding phage were first eliminated on GST-coated wells and the unbound phage preparations were incubated on α_M I domain GST fusion protein-coated wells. The α_M I domain-binding phage were enriched by three rounds of panning and the peptide sequences were determined. With the exception of one linear peptide, the peptides were derived from the cyclic CX_7C and CX_8C libraries. The I domain-binding sequences showed only one conserved motif, a somewhat unexpected finding in terms of the known ligand binding promiscuity of the I domain. The bound peptides contained two consecutive negatively charged amino acids, i.e. glutamatic and/or aspartatic acids, followed by glycine and tryptophan residues (Fig. 1A). The consensus D/E-/D/E-G/L-W determined by this approach was clearly different from LLG-C4 and other β_2 integrin-binding peptides reported so far.

We first prepared the phage display peptides as intein fusion proteins, from which the peptides were cleaved. This allowed us to rapidly test the peptide solubility and the binding specificity before large-scale chemical peptide synthesis. The peptides were cloned using oligonucleotide primers that amplify the peptide library insert from the phage vector. Consequently, all the peptides prepared contain the vector-derived sequences ADGA and GAAG in the NH_2- and COOH- termini, respectively. Phage binding experiments using soluble peptides as competitors indicated that the peptides bearing the two adjacent negative charges bound to a common site (not shown). We chose the peptide ADGA-CILWMDDGWC-GAAG (DDGW) for further experiments as this peptide showed strong binding and was highly soluble in aqueous buffers (soluble in 50 mM NaOH at >10 mM concentrations). The peptide was also prepared

by chemical synthesis. The phage bearing the DDGW sequence avidly bound to the α_M I domain and this was readily inhibited by low concentrations of the DDGW peptide, but only marginally affected by the LLG-C4 peptide, indicating different binding sites for DDGW and LLG-C4 (Fig. 1B). Control phage bearing other peptide sequences did not bind. The DDGW-bearing phage also showed also specific binding to the α_L I domain that was inhibitable by DDGW but the interaction was weaker than with the α_M I domain (Fig. 1C and data not shown). No binding was observed with the α_X I domain or GST used as a control (Fig. 1C).

Characterization of DELW sequence on the catalytic domain of gelatinases that mediates interaction with the β_2 integrin I domains

We searched protein databases for matches to the novel D/E-/D/E-G/L-W motif. One of the phage library-derived peptides, CPEELWWLC, was highly similar to the DELW(S/T)LG sequence present on the catalytic domain of MMP-2 and MMP-9 gelatinases (Fig. 1A). DELW-like sequences with double negative charges are also present in other secreted MMPs but not in the membrane-type MMPs such as MMP-14.

No MMP has been reported to bind to the leukocyte β_2 integrins. We therefore set out to study whether MMP-9 in particular could be a ligand of the β_2 integrins as MMP-9 gelatinase is the major leukocyte MMP and is induced during β_2 integrin activation. As a first step, we synthesized the whole proMMP-9 sequence as overlapping 20-mer peptides on a pepspot membrane. Binding assays with the α_M I domain revealed a single active peptide that located to the MMP-9 catalytic domain (Fig. 1D). No binding was observed, when the I domain was omitted and the membrane was probed with antibodies only. The sequence of the I domain-binding peptide was QGDAHFDD**DELWSLG**KGVVV and it contained the binding motif identified by phage display (Fig. 1D).

The active MMP-9 peptide contained four consecutive amino acids with negative charges, DDDE. To study the importance of these residues, the aspartic and glutamic acid residues that were closest to the tryptophan were replaced by alanines. At the same time the peptide length was shortened to 15-mer. The alanine mutagenesis

significantly abrogated I domain binding on the pepspot filter; the OD value dropped from 2010 to 476 (Table I). To study whether the negatively charged peptide from other MMPs is also active, we synthesized the corresponding 15-mers and the double alanine mutations. Sequences from MMP-1, 2, 3, 7, 8, 9 and 13, but not the membrane-anchored MMP-14 (MT1-MMP), could bind the α_M I domain. Alanine mutations always decreased the binding.

We did similar pepspot analysis for some of the known I domain ligands, which contain D/E-/D/E-G/L-W like sequences. Peptides derived from myeloperoxidase, catalase, thrombospondin-1 and complement protein iC3b strongly bound the I domain in this assay and the double alanine mutation caused a loss of binding (see Table I). Of the three iC3b peptide permutations tested, ARSNLD**ED**IIAEENI was the active one. The acidic residues were followed by a hydrophobic isoleucine cluster in this peptide. The soluble DDGW peptide efficiently inhibited the binding of this peptide to the I domain. Weaker I domain binding was observed with one complement factor H-derived peptide and one fibronectin-derived peptide. Peptides derived from ICAMs-1, 2 and 3, neutrophil inhibitory factor, Cyr61, fibrinogen, GP1b, factor X, or E-selectin lacked activity.

Alanine scanning mutagenesis of the DDGW peptide with the pepspot system similarly indicated the importance of the glutamic acid residues for I domain binding (Fig. 1E). Alanine mutations of the glycine or either one of the tryptophan residues also inactivated the peptide. Mutations of the isoleucine, leucine or methionine residues were tolerated. Deletion of the ADGA sequence from the N terminus had no effect on I domain binding, but removal of the C terminal GAAG sequence abolished the binding. As the peptides were immobilized via the C terminus on the filter, a sufficient linker sequence such as GAAG seemed important. We also tested a series of truncated cyclic peptides to identify the shortest active sequence. This analysis showed that ADGA-CEDGWC-GAAG, but not ADGA-CDDGWC-GAAG was the minimal peptide that supported α_M I domain binding. The longer side chain of glutamate compared to aspartate is probably required to bring the negatively charged carboxyl group in the correct position for I domain binding.

Progelatinases bind to purified $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins and their I domains

We next used a microtiter well-based sandwich assay to study gelatinase binding to purified integrins. Progelatinases bound in a concentration-dependent manner to coated $\alpha_M\beta_2$ integrin (Fig. 2A). Curiously, MMP-2 and MMP-9 lost the integrin binding ability after activation by trypsin or APMA. The binding of proMMP-2 and proMMP-9 was observed with both $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins and their corresponding I domains (Fig. 2B and 2C). No binding was detected on the α_X I domain or the $\alpha_1\beta_1$ and $\alpha_3\beta_1$ integrins.

The DDGW peptide was an efficient inhibitor and it inhibited proMMP-9 binding to the α_M I domain with an IC_{50} of 20 μM (Fig. 3A and 3B). To demonstrate that the negative charges of aspartic acids are essential for the peptide activity, the peptide ADGACILWMKKGWCGAAG (KKGW) containing lysines in place of aspartic acids was prepared. As expected, the KKWG peptide was inactive and did not compete with proMMP-9 binding. We were also interested in testing lovastatin, as its binding site in the α_L I domain is known (34,35). Lovastatin was not able to compete with proMMP-9 even at a high concentration.

ProMMP-9 bound like a true integrin ligand, as the cation chelator EDTA (5 mM) nearly completely prevented the binding (Fig. 3C and 3D). For background measurement in the sandwich assay, we used antibodies alone (control), or coating with ICAM-1 or wild type GST. The gelatinase-binding peptide CTT (200 μM) inhibited proMMP-9-integrin interaction with the same efficiency as EDTA did. The control peptides STTHWGFTLS (STT) and CTTHAGFTLC (W→A CTT), which lack gelatinase inhibitory activity (26), were without effect. A non-peptide chemical MMP inhibitor (Inh1) also prevented proMMP-9 binding. As EDTA inhibits both the gelatinase and the integrin, we used integrin blocking antibodies and ligand peptides to demonstrate the specific binding activity of β_2 integrin. The known ligand-binding blocking antibodies MEM 170, MEM 83, and LM2/1 inhibited proMMP-9 binding. A control antibody TL3 had no effect. The I domain binding peptide LLG-C4 showed a partial inhibitory effect. RGD-4C, a ligand of α_V integrins, served as control peptide and had no effect on proMMP-9 binding. The purity of the integrins was typically

more than 90% and that of I-domains 95%, making it unlikely that progelatinases would bind to impurities in the preparations.

Progelatinase-integrin complexes were also obtained by co-precipitation experiments using HT1080 conditioned medium as a source of proMMP-9 and proMMP-2, which were analyzed by zymography. The progelatinases co-precipitated with $\alpha_M\beta_2$ integrin or α_M I domain GST fusion protein when these were used as a bait. The integrin added to the medium was immunoprecipitated with the α_M antibody OKM10 (Fig. 4A). The α_M I domain GST protein was pulled down with glutathione-beads (Fig. 4B). CTT but not STT had an inhibitory effect. Inhibition of the I domain by LM2/1, ICAM-1 or LLG-C4 also affected the pull-down of progelatinases. GST control did not coprecipitate the gelatinases. No active forms of gelatinases were found to coprecipitate with $\alpha_M\beta_2$ or the I domain, when APMA-treated HT-1080 medium or APMA-activated MMP-9 was used (not shown).

As the gelatinase inhibitors CTT and Inh1 prevented the binding of proMMP-9 to the integrin, it can be anticipated that CTT and Inh1 avidly bind to proMMP-9. To gain more insight into this, we examined binding of proMMP-9 to immobilized CTT peptide. ProMMP-9 specifically bound to the CTT-GST fusion protein (Fig. 5A) but not to LLG-C4-GST. CTT and Inh1 at 100 μM concentrations effectively competed in binding but W→A CTT did not. The proMMP-9 preparation did not contain detectable amounts of active MMP-9 on zymography analysis, and after proMMP-9 activation with APMA, the CTT-GST binding increased. CTT and Inh1 could also bind to proMMP-9 secreted into the medium of PDBu-activated THP-1 leukemic cells (Fig. 5B) or HT1080 fibrosarcoma cells (not shown). A time-dependent reduction in the gelatinolytic activity of proMMP-9 was observed with CTT (panel 1) and Inh1 (panel 3), but not with the W→A CTT peptide (panel 4). Western blot analysis indicated that CTT does not decrease the secretion of proMMP-9 by the cells (panel 2). Furthermore, the CTT complex was reversible and disappeared after repeated freezing and thawing of the samples.

Demonstration of a cell-surface complex between progelatinases and β_2 integrins

To study whether the progelatinases occur in a complex with the β_2 integrins on the leukocyte surface, we performed immunoprecipitation and co-localization studies. First, we examined THP-1 monocytic leukemia cells in the resting state and after induction by PDBu, which mimics leukocyte activation *in vivo*. THP-1 cell-stimulation with PDBu led to upregulation of MMP-9 (data not shown). The cell surface glycoproteins of THP-1 cells were labelled with tritium [^{3}H] followed by immunoprecipitation with β_2 integrin and MMP-9 antibodies. In the PDBu-activated cells, the α_M chain antibody OKM10 and β_2 chain antibody 7E4 immunoprecipitated two [^{3}H]-labelled proteins corresponding to the integrin α_M chain (165 kDa) and β_2 chain (95 kDa) (Fig. 6A, lanes 9-10). Importantly, polyclonal MMP-9 antibodies immunoprecipitated the same two integrin chains (lane 7). In non-activated cells, essentially no co-precipitation of α_M and β_2 were observed with MMP-9 antibodies, although the α_M and β_2 chains were present. The co-precipitation of the integrin chains by MMP-9 antibodies was prevented by the CTT peptide (lane 8). The control antibody (TL3) did not precipitate any proteins.

With the [^{3}H]-labelled cells, we did not observe any band corresponding to proMMP-9, perhaps because the carbohydrates of proMMP-9 are poorly labelled. We therefore analyzed the PDBu-activated THP-1 cells by Western blotting (Fig. 6B). ProMMP-9 was readily immunoprecipitated with antibodies against MMP-9, α_M or α_L, but not by the control antibody. MMP-9 antibodies in turn were able to immunoprecipitate the α_M but not the α_L chain. MMP-2 antibodies similarly co-precipitated α_M but not α_L. When the cell lysate was precleared with the α_M antibody OKM-10, the amount of immunoprecipitated α_M and proMMP-9 clearly decreased (lane 6). Preclearing with the α_L antibody TS2/4 did not significantly remove α_M or proMMP-9, but abolished the α_L precipitation (lane 7).

As THP-1 cells do not express high amounts of the α_L chain (20), we examined the Jurkat T cell line, which expresses more α_L than α_M (28). We observed a significant immunoprecipitation of α_L by MMP-9 and MMP-2 antibodies after PDBu activation (Fig. 6C). Furthermore, the α_L antibody co-precipitated more proMMP-9 in

comparison to the α_M antibody. No proMMP-9 co-precipitated with MMP-2 antibodies in Jurkat or THP-1 cells.

ProMMP-9 and $\alpha_M\beta_2$ were found to co-localize on the cell surface following PDBu-activation of THP-1 cells as studied by fluorescence and confocal microscopy (Figs. 7A and 7B, respectively). Using a higher magnification, colocalization was primarily seen in cell surface clusters (Fig. 7B), and to a lesser extend on areas where cells contacted each other (not shown). We believe that the MMP-9 co-localizing with $\alpha_M\beta_2$ is the proMMP-9, as the activated MMP-9 did not bind to $\alpha_M\beta_2$. Without PDBu activation, there was hardly any co-localization of proMMP-9 and $\alpha_M\beta_2$. The secondary antibodies did not stain the cells when the primary antibodies were omitted (data not shown). When the cells were preincubated with the CTT peptide or recombinant soluble ICAM-1 to block proMMP-9 or $\alpha_M\beta_2$, the cell surface clusters did not form and the proMMP-9-$\alpha_M\beta_2$ colocalization was not observed (not shown). ProMMP-9-$\alpha_M\beta_2$ colocalization was also observed on Jurkat cells following phorbol ester stimulation (not shown).

Blocking the progelatinase/β_2 integrin complex with DDGW releases cell-bound proMMP-9 and inhibits cell migration but not adhesion

As the DDGW peptide is an integrin ligand, one of the questions was whether it can support adhesion of leukocytes. We studied adhesion of human myelomonocytic THP-1 cells on immobilized glutaraldehyde-polymerized peptide. Phorbol-ester activated cells efficiently bound to the DDGW peptide, whereas there was no binding in the absence of cell activation (Fig. 8A). As a positive control, the recombinant intein-produced ADGA-CPCFLLGCC-GAAG peptide supported adhesion, but unlike the DDGW peptide, it also supported adhesion in the absence of integrin activation. The acute myeloid leukemic cell line OCI/AML-3 also avidly adhered to DDGW, whereas human fibrosarcoma HT1080 cells which lack β_2 integrins did not (not shown). As THP-1 cells were able to adhere on DDGW, we next studied the effect of the peptide on β_2 integrin dependent adhesion to fibrinogen and ICAM-1. Interestingly, DDGW did not block cell adhesion to fibrinogen, whereas the LLG-C4 peptide blocked the adhesion as previously reported (Fig. 8B). Similarly, DDGW did not block the binding of recombinant α_M I-domain to immobilized fibrinogen (not

shown). DDGW did not either block cell adhesion on ICAM-1-Fc fusion protein. As a control, the blocking antibody 7E4 against β_2 integrins prevented the ICAM-1 binding, indicating that the THP-1 cells bound in a β_2 integrin dependent manner (Fig. 8C). We also found no blocking effect of DDGW on THP-1 adhesion to LLG-C4-GST fusion protein (not shown).

The second question raised by these studies was whether the DDGW peptide can release cell-bound proMMP-9. When THP-1 cells were cultured for 48 h in the presence of DDGW, an increase of proMMP-9 level was observed in the conditioned medium as studied by gelatin zymography (Fig. 8D). The peptide increased both monomeric and dimeric proMMP-9 in the culture medium. In contrast, CTT slightly decreased or inhibited active proMMP-9. KKGW and W→A CTT had no effect.

Finally, we studied the role of the progelatinase/β_2 integrin complex in leukocyte migration using a transwell assay in which leukocyte migration can be adjusted by the choice of coated matrix or ligand protein. We tested that the CTT, LLG and DDGW peptides are not toxic to the THP-1 cells in a 48 h time frame at >200 μM concentrations using an MTT assay. Using transwells coated with 10% serum in cell culture medium, we first studied the effect of peptides on the basal migration of THP-1 cells in the absence of any stimulus by phorbol ester or an adhesive matrix. Under such conditions, CTT, LLG-C4 or the gelatinase inhibitor Inh1 at a 200 μM concentration had no effect on THP-1 migration indicating no active involvement of gelatinases or β_2 integrins (Fig. 9A). We have previously shown that when the transwells are coated with LLG-C4-GST fusion protein, THP-1 cells adhere and migrate in a β_2 integrin dependent manner (14). Thus, transwells were coated with LLG-C4-GST fusion protein or GST alone. Both the DDGW and CTT peptide, but not KKGW, inhibited the migration of THP-1 cells on the LLG-C4-GST substratum (Fig. 9B). The soluble LLG-C4 peptide also blocked the migration. In the presence of GST coating, cell migration was neglible. To verify that the effect of DDGW peptide was β_2 integrin dependent, HT1080 fibrosarcoma cells lacking these integrins were allowed to migrate in the presence of CTT, DDGW, KKGW or LLG-C4. Of these peptides, only CTT was capable of inhibiting cell migration (Fig. 9C).

DISCUSSION

Analysis of α_M I domain-binding peptides led to the finding that MMPs, particularly the MMP-9 and MMP-2 progelatinases, are potent β_2 integrin ligands. Our studies show that proMMP-9, the major MMP of activated leukocytes, is co-localized with the β_2 integrin on the cell surface. Cell surface labelling and co-immunoprecipitation further demonstrates the occurrence of the complex in leukemic cell lines. Finally, we have found evidence that this proteinase-integrin complex plays a role in migration of the leukemic cells.

Although phage display has been extensively used with whole integrins (14,36-44), to the best of our knowledge, this is the first successful phage display selection on an isolated integrin I domain. We could enrich only one binding motif even though the α_M I domain can bind a variety of ligands (1-3). The peptide motif we isolated could not compete with the ICAM-1, fibrinogen, or LLG-C4 ligands. The success of phage display depends on the libraries used and the biopanning conditions. Our method favoured "high affinity" interactions with the cyclic peptides yielding the D/E-D/E-G/L-W motif. Interestingly, this motif shows a high degree of similarity to the CWDD(G/L)WLC peptide isolated by phage display as an RGD sequence-binding peptide (40). By recognizing the RGD ligand sequence, CWDDGWLC structurally and functionally behaves like a minimal integrin. Here, we have identified the DDGW peptide in a reverse situation, as a ligand to integrin. However, the RGD sequence does not compete with the α_M I domain as the GRGDSP peptide at a 1 mM concentration was unable to inhibit proMMP-9 binding to the I domain[2]. It will be interesting to see whether the DDGW peptide recognizes a positively and negatively charged sequence in the I domain.

Some hints for the binding site of DDGW come from the interaction of iC3b with $\alpha_M\beta_2$ integrin. Our pepspot analysis showed that the iC3b peptide ARSNLDEDIIAEENI, but not the control peptide ARSNLDAAIIAEENI, bound the α_M I domain and the DDGW peptide blocked this binding. The DEDIIAEENI

sequence with multiple adjacent negative charges is required for efficient binding of iC3b to $\alpha_M\beta_2$ integrin (45). The binding site of complement protein iC3b in the I domain has been mapped indicating a role for the positively charged amino acid residue K^{245} for iC3b binding (46). Mutation of this residue does not affect the binding of the fibrinogen recognition peptide (47). This may account for the inability of the DDGW peptide to inhibit ICAM-1 and fibrinogen-mediated cell adhesion. These findings suggest that the K^{245} residue is the positively charged contact site for the DDGW peptide and the D/E-D/E-G/L-W motif as well.

The pepspot analysis indicates that a class of β_2 integrin ligands contains an active D/E-D/E-G/L-W motif. These include the previously identified $\alpha_M\beta_2$ ligands iC3b, thrombospondin-1, and the enzymes myeloperoxidase and catalase (3,4,5,48). In our experiments, the peptides derived from several secreted MMPs, but not membrane-bound MT1-MMP, were also active. It is notable that the D/E-D/E-G/L-W motif is relatively conserved in the secreted members of the MMP family. Whether other MMPs in addition to the progelatinases can make β_2 integrin complexes, remains to be determined.

Finding of a dominant integrin-binding site in the catalytic domain of proMMP-9 was unexpected, because previous studies suggested an essential role for another MMP domain, the hemopexin domain, in integrin binding. The hemopexin domains mediated MMP-2 binding to the $\alpha_V\beta_3$ integrin (49-51) and MMP-1 binding to the $\alpha_2\beta_1$ integrin (52,53). The cleaved hemopexin domain of MMP-2 has also been shown to occur in vivo and to inhibit angiogenesis (50). Understandably, phage peptide display and pepspot techniques have limitations and only linear peptide sequences can be analysed, not protein conformations. Thus, in the present study we cannot make conclusions of the function of separate MMP-9 domains in integrin binding and it remains to be seen whether cleavage products of MMP-9, if present in vivo, can act as β_2 integrin ligands. Our studies suggest that the peptide sequence from the catalytic domain is essential for the binding of full-length proMMP-9 to the β_2 integrin, as the synthetic DDGW peptide could completely inhibit the integrin binding. It was important to use natural proMMP-9 because $\alpha_M\beta_2$ is known to bind to denaturated proteins and this may sometimes be the case for bacterially expressed proteins (4).

Furthermore, we did not observe binding of an active MMP-2 or MMP-9 to the integrin, although the DELW(T/S)LG sequence should remain unchanged in the active enzyme. We rather found that AMPA and trypsin, activators of proMMP-9, released MMP-9 from THP-1 cells, apparently affecting the integrin complex[3]. These results suggest that the proenzyme presents the integrin binding site more efficiently than the active enzyme and the β_2 integrin may even control the activation of the proenzyme.

In the three-dimensional structures of proMMP-2 and –9 (54,55), the I domain binding site is located in the vicinity of the zinc-binding catalytic sequence HEFGHALGLDH between the catalytic domain and the fibronectin type II repeats. This location suggests a mechanism for evading proMMP-9 inhibition by tissue inhibitors of MMPs (TIMPs) or α_2 -macroglobulin. In the absence of inhibitors, the cell surface-localized proMMP-9 would be readily susceptible for activation and substrate hydrolysis, which may also occur in the presence of intact propeptide (56). On the other hand, because the binding site of the I domain is located in the vicinity of the catalytic groove, it also suggests an explanation for the blocking of MMP-9/β_2 integrin interaction by the small molecule MMP inhibitors such as CTT and Inh1.

The activity of the DDGW peptide in the THP-1 cell migration assay suggests an important function for the integrin-progelatinase complex in leukocyte migration. Obviously, we cannot exclude the possibility that the DDGW peptide blocks binding of other ligands than gelatinases and in this way inhibits the leukocyte migration. However, as the specific gelatinase inhibitor CTT also blocks the THP-1 cell migration, these results strongly suggest that the proMMP-9/β_2 integrin complex is the main target for DDGW. Interestingly, the DDGW peptide blocked THP-1 cell migration although it increased the level of proMMP-9 in the medium, which suggests that cell-surface bound rather than total MMP-9 level is a critical factor in cell migration.

FIGURE LEGENDS

FIG. 1. **Identification of an I domain binding site in progelatinase.** A, Phage display peptide sequences specifically bound to the α_M I domain. The consensus motif is shown in bold. Peptides with the strongest binding (CILWMDDGW) and the highest similarity (CPEELWWLC) are aligned with human MMPs (accession numbers shown in parenthesis). B, Phages bearing the CILWMDDGWC peptide or a control peptide were allowed to bind to immobilized α_M I domain-GST fusion protein (20 ng/well) in the absence or presence of 15 μM DDGW peptide or LLG-C4 peptide. Bound phages were detected using a monoclonal anti-M13 phage antibody. Mean absorbance of triplicate samples ± SD is shown. C, α_L, α_M, or α_X I domain-GST fusions were coated on microtiter wells as in B, and the binding of CILWMDDGWC peptide bearing phage or a control phage was measured. D, Peptides covering the complete sequence of proMMP-9 were synthesized as overlapping peptides on a pepspot membrane. The α_M I domain (0.5 μg/ml) was allowed to bind to the peptides followed by immunodetection using anti-α_M I domain antibody LM2/1. The α_M I domain-binding peptide 13 (arrow) is shown in boldface and the zinc binding catalytic sequence is underlined. The prodomain (Pro), catalytic domain containing the fibronectin type II repeats (Cat) and hemopexin domain (Pex) are marked to illustrate the domain structure of proMMP-9. E, Alanine-mutated and truncated peptides were synthesized on a pepspot filter and probed with the recombinant α_M I domain (5μg/ml). Bound I domain was measured using mAb MEM-170 (5μg/ml) followed by HRP-conjugated anti-mouse secondary antibody and ECL detection. The binding was quantified by densitometric scanning. The bars show α_M I domain binding to single peptide spots as arbitrary optical density units/mm^2. Similar results were obtained in three independent experiments.

FIG. 2. **Binding of progelatinases to purified integrins and I-domains.** A, proMMP-9, proMMP-2 or their trypsin-activated forms, at the concentrations indicated, were allowed to bind to $\alpha_M\beta_2$ integrin-coated wells. Appropriate MMP antibodies were used to determine binding. The results in this and other figures are

represented as the means ± SD from triplicate wells. B, binding of proMMP-9 (80 ng/well) was examined on microtiter wells coated with an integrin ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_1\beta_1$, $\alpha_3\beta_1$) or an I-domain (α_L, α_M, α_X). The binding was determined using anti-MMP-9 antibody. C, proMMP-2 (80 ng/well) was allowed to bind to $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_1\beta_1$ or the I-domains α_L, α_M or α_X. The binding was determined using an anti-MMP-2 antibody.

FIG. 3. **Inhibitors of the proMMP-9 / β_2 integrin complex.**
A, α_M and α_L I domain-GST fusions were immobilized on microtiter wells. ProMMP-9 (100 ng/well) was added in the presence or absence of various peptides (200 μM) or lovastatin (100 μM) in a buffer containing 0.5% BSA. ProMMP-9 binding was detected using a monoclonal antibody against MMP-9. The results are shown as percent binding compared to binding in the absence of inhibitors (100%) and no proMMP-9 added (0%). B, DDGW peptide blocks proMMP-9 binding to α_M in a dose dependent manner. The assay was done similarly as in (A), except various concentrations of peptides were added to compete for binding. All samples were assayed as triplicates and results shown are means ± SD from a representative experiment. C, proMMP-9 binding to $\alpha_M\beta_2$ and $\alpha_L\beta_2$ was examined in the absence and presence of EDTA (5 mM), MMP inhibitor-1 (100 μM), CTT (200 μM), STT (200 μM), MEM170 (40 μg/ml), or control TL3 antibody (40 μg/ml). The background of primary and secondary antibodies was measured by omitting proMMP-9 from the wells or by coating with ICAM-1. D, proMMP-9 binding to purified α_M and α_L I-domain GST fusion proteins or wild type GST was studied in the absence or presence of competitors as indicated. Control shows the background when proMMP-9 was omitted.

FIG. 4. **Coprecipitation of progelatinase with β_2 integrin.** A, $\alpha_M\beta_2$ integrin (3 μg) was incubated with a 500 μl sample of HT1080 medium containing proMMP-9 and proMMP-2 in the absence or presence of CTT or STT (200 μM) for 2 h. The integrin was immunoprecipitated with the OKM10 antibody, and the immunoprecipitates were analyzed by gelatin zymography. In control experiments, integrin was omitted from the medium and ICAM-1 was added instead. B, a 500 μl sample of HT1080 medium containing proMMP-9 and proMMP-2 was incubated with the α_M I-domain GST (3μg) or LLG-C4-GST control. ICAM-1, LM2/1, CTT, STT, or LLG-C4 were used as

competitors. GST was pulled down with glutathione beads, and bound proteins were analyzed by zymography. The lane 1 in the figure insert: the proMMP-2 and proMMP-9 zymogens present in non-treated HT1080 medium, lane 2: lack of gelatinases pulled down with control LLG-C4-GST, lane 3: proMMP-9 and proMMP-2 coprecipitated by α_M I domain GST fusion protein.

FIG. 5. **CTT peptide binds to both latent and active MMP-9.**
A, Binding of proMMP-9 or APMA-activated MMP-9 to CTT-GST was examined in the absence or presence of competitors CTT (100 μM), W→A mutant CTT (100 μM), and Inh1 (100 μM). GST control was LLG-C4-GST. Binding was determined as in Figs. 2 and 3. The background in the absence of proMMP-9 is shown. D, THP-1 cells were incubated in serum-free medium containing CTT, Inh1 or W→A CTT at 200 μM concentration. Samples from the media were collected at the time points indicated and analyzed by zymography (panels 1, 3, and 4) or Western blotting (panel 2).

FIG. 6. **Progelatinases occur in complex with $\alpha_M\beta_2$ and $\alpha_L\beta_2$ in PDBu-activated THP-1 and Jurkat cells.** A, THP-1 cell surface proteins were [^{3}H]-labelled using periodate-tritiated borohydride and analyzed by immunoprecipitation. CTT was used as a competitor (200 μM). The immunoprecipitated samples were resolved on a 8-16% polyacrylamide gel, and the film was exposed for 3 days. Lanes 1-4 are from non-activated cells and lanes 6-10 from PDBu-activated cells. Lane 5 shows molecular weight markers. B, lysates from PDBu-activated THP-1 cells were immunoprecipitated with integrin or MMP antibodies followed by Western blotting with α_M (OKM10), α_L (TS2/4) or MMP-9 antibodies. Preclearings of the cell lysates were done using α_M (lane 6) and α_L (lane 7) antibodies. C, lysates from PDBu-activated Jurkat cells were subjected to immunoprecipitation followed by blotting with the α_L (MEM83) and MMP-9 antibodies.

FIG. 7. **PDBu-induced colocalization of $\alpha_M\beta_2$ and proMMP-9 in THP-1 cells.** Cells were preincubated for 30 min at +37°C with 50 nM PDBu. A, cells were treated with anti-α_M OKM10 and anti-MMP-9 antibodies followed by FITC-labeled (green fluorescence) and TRITC-labeled (red fluorescence) secondary antibodies. Yellow color indicates colocalization of $\alpha_M\beta_2$ and proMMP-9. Bars, 8.5 μm. B,

immunofluorescence staining shows intense colocalization of MMP-9 (polyclonal antibody) and $\alpha_M\beta_2$ integrin (OKM-10) on the surface of PDBu-activated THP-1 cells at higher magnification as visualized by confocal microscopy (Bars, 2.5 µm).

FIG. 8. **The DDGW peptide supports THP-1 cell adhesion and induces proMMP-9 release, but does not block adhesion to the major β_2 integrin ligands fibrinogen and ICAM-1.** A, THP-1 cells were allowed to bind to immobilized, glutaraldehyde polymerized peptides with or without phorbol ester activation (50 nM) and the adherent cells were quantitated by phosphatase assay. THP-1 cells were allowed to bind to immobilized fibrinogen B, or recombinant ICAM-1-Fc C, in the presence or absence of 200 µM soluble peptides. All samples were assayed as triplicates and results show means ± SD. Identical results were obtained in two other independent experiments. D, THP-1 cells were incubated in the presence or absence of peptides at 200 µM concentration for 48 hours. Aliquots of conditioned medium were analyzed by gelatin zymography. Arrows show the 92 kDa proMMP-9 and 220 kDa proMMP-9 dimer.

FIG. 9. **Peptide inhibition of THP-1 cell migration.** THP-1 cells were preincubated with the peptide as indicated at a 200 µM concentration for 1 h at room temperature and applied to transwells in the absence A, or presence B, of LLG-C4-GST coating. Cells were allowed to migrate for 16 hours at +37°C. Cells migrated to the lower surface of the filter were stained and counted microscopically. C, HT1080 fibrosarcoma cell migration was similarly assayed in the absence of LLG-C4-coating. The bars show means ± SD from triplicate wells.

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Footnotes

[1]The abbreviations used are: APMA, aminophenylmercuric acetate; $\alpha_M\beta_2$, CD11b/CD18, Mac-1 integrin; CTT, CTTHWGFTLC peptide; DDGW, ADGACILWMDDGWCGAAG peptide; GST, glutathione-S-transferase; ICAM, intercellular adhesion molecule; Inh1, matrix metalloproteinase inhibitor 1; KKGW, ADGACILWMKKGWCGAAG peptide; LLG-C4, CPCFLLGCC peptide; MMP, matrix metalloproteinase; RGD-C4, ACDCRGDCFCG peptide; STT, STTHWGFTLC peptide; W→A CTT, CTTHAGFTLC peptide.

[2]M.B. and E.K, unpublished observations.

[3]M.S. and E.K, unpublished observations.

Table I: Pepspot analysis of peptides derived from MMPs and $\alpha_M\beta_2$ integrin ligands

Protein	Peptide	OD/mm²	Binding positivity
MMP-1	DAHFDEDERWTNNFR	1792	+
	DAHFDE**AA**RWTNNFR	1417	-
MMP-2	DSHFDDDELWTLGEG	4687	+++
	DSHFDD**AA**LWTLGEG	3334	++
MMP-3	DAHFDDDEQWTKDTT	4993	+++
	DAHFDD**AA**QWTKDTT	2188	+
MMP-7	DAHFDEDERWTDGSS	4043	++
	DAHFDE**AA**RWTDGSS	2065	+
MMP-8	DAHFDAEETWTNTSA	4295	++
	DAHFDA**AA**TWTNTSA	1258	-
MMP-9	DAHFDDDELWSLGKG	2010	+
	DAHFDD**AA**LWSLGKG	476	-
MMP-13	DAHFDDDETWTSSSK	4324	++
	DAHFDD**AA**TWTSSSK	1732	+
MMP-14	DTHFDSAEPWTVRNE	1264	-
	DTHFDS**AA**PWTVRNE	1198	-
MMP-1	SGDVQLDDIDGIQAI	484	-
	SGDVQL**AA**IDGIQAI	441	-
MMP-3	RFRLSQDDINGIQSL	810	-
	RFRLSQ**AA**INGIQSL	1541	+
MMP-8	NYSLPQDDIDGIQAI	3348	++
	NYSLPQ**AA**IDGIQAI	505	+
MMP-13	HFMLPDDDVQGIQSL	542	-
	HFMLPD**AA**VQGIQSL	384	-
MMP-14	NFVLPDDDRRGIQQL	518	-
	NFVLPD**AA**RRGIQQL	609	-
Fibronectin	HEATCYDDGKTYHVG	1271	-
	HEATCY**AA**GKTYHVG	596	-
ICAM-3	LNATESDDGRSFFCS	369	-
	LNATES**AA**GRSFFCS	277	-
Complement factor H	EEMHCSDDGFWSKEK	2972	+
	EEMHCS**AA**GFWSKEK	321	-
TSP-1	WPSDSADDGWSPWSE	4655	+++
	WPSDSA**AA**GWSPWSE	1543	+
NIF	DPVCIPDDGVCFIGS	221	-
	DPVCIP**AA**GVCFIGS	114	-
ICAM-2	NSTADREDGHRNFSC	75	-
	NSTADR**AA**GHRNFSC	73	-
Fibronectin	NVYQISEDGEQSLIL	1260	-
	NVYQIS**AA**GEQSLIL	453	-
Fibronectin	VTYSSPEDGIHELFP	301	-
	VTYSSP**AA**GIHELFP	212	-
Cyr61	KMRFRCEDGETFSKN	317	-

	KMRFRC**AA**GETFSKN	386 -
Myeloperoxidase	WLPAEYEDGFSLPYG	4603 +++
	WLPAEY**AA**GFSLPYG	924 -
Catalase	AVKFYTEDGNWDLVG	5045 +++
	AVKFYT**AA**GNWDLVG	690 -
Fibrinogen alpha	KEVVTSEDGSDCPEA	124 -
	KEVVTS**AA**GSDCPEA	225 -
Fibrinogen beta	RKQCSKEDGGGWWYN	483 -
	RKQCSK**AA**GGGWWYN	325 -
Fibrinogen alpha	GFGSLNDEGEGEFWL	732 -
	GFGSLN**AA**GEGEFWL	397 -
GP1b	GCPTLGDEGDTDLYD	544 -
	GCPTLG**AA**GDTDLYD	238 -
ICAM-1	VSVTAEDEGTQRLTC	51 -
	VSVTAE**AA**GTQRLTC	68 -
Factor X	DRNTEQEEGGEAVHE	379 -
	DRNTEQ**AA**GGEAVHE	236 -
E-selectin	TCTFDCEEGFELMGA	309 -
	TCTFDC**AA**GFELMGA	70 -
E-selectin	SCNFTCEEGFMLQGP	138 -
	SCNFTC**AA**GFMLQGP	54 -
E-selectin	SCAFSCEEGFELHGS	236 -
	SCAFSC**AA**GFELHGS	74 -
Fibronectin	TFHKRHEEGHMLNCT	39 -
	TFHKRH**AA**GHMLNCT	12 -
Fibronectin	VEYELSEEGDEPQYL	3676 ++
	VEYELS**AA**GDEPQYL	2682 +

Range: 0-1499 OD/mm2 = -, 1500-2999 = +, 3000-4499 = ++, >4500 = +++

	Peptide	No competitor	Soluble DDGW
DDGW peptide	ADGACILWMDDGWCGAAG	10859	0
iC3b	ARSNLDEDIIAEENI	13265	409
	ARSNLD**AA**IIAEENI	0	0
iC3b	EDIIAEENIVSRSEF	0	0
	EDIIA**AA**NIVSRSEF	0	0
iC3b	EGVQKEDIPPADLSD	0	0
	EGVQK**AA**IPPADLSD	0	0

CLAIMS

1. A compound inhibiting the leukocyte progelatinase/β_2-integrin complex, the compound comprising the tetrapeptide motif D/E-D/E-G/K-W.

2. The compound according to claim 1, wherein the tetrapeptide motif is DDGW.

3. The compound according to claim 1 or 2 for use as a pharmaceutical.

4. The compound according to claim 1 or 2 for use in inhibiting leukocyte migration.

5. A pharmaceutical composition comprising the compound according to claim 1 or 2, and a pharmaceutically acceptable carrier.

6. Use of the compound according to claim 1 or 2 for the manufacture of a pharmaceutical composition for the treatment of conditions dependent on leukocyte migration.

7. Use according to claim 6 for the manufacture of a pharmaceutical composition for inhibiting the adhesion of progelatinases to β_2-integrins.

8. The compound according to claim 1 or 2 for use in prevention of inflammatory conditions.

9. The compound according to claim 1 or 2 for use in prevention of leukaemia cell migration.

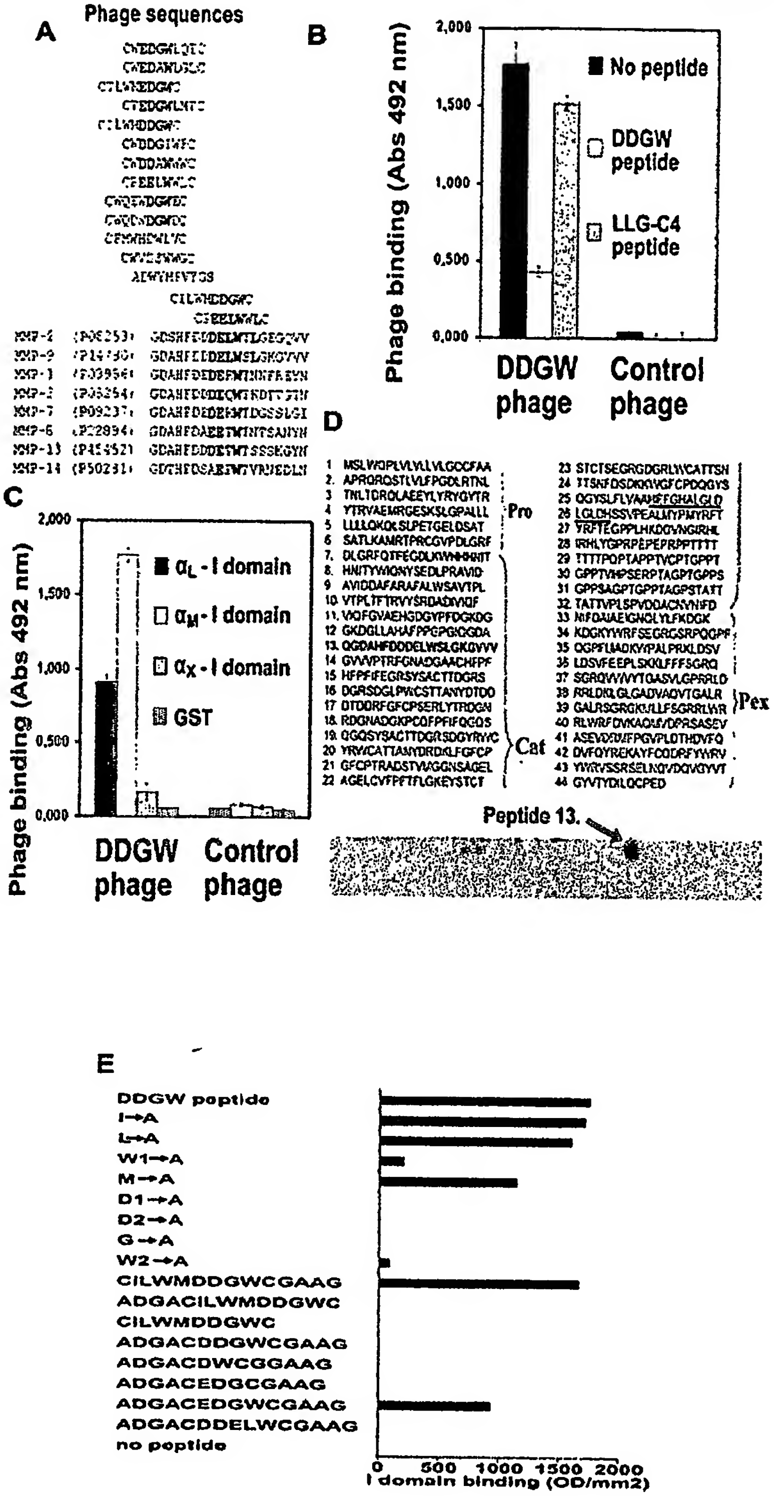
10. The compound according to claim 1 or 2 for use in treatment of leukaemia.

SUMMARY

The $\alpha_M\beta_2$ integrin of leukocytes can bind a variety of ligands. We screened phage display libraries to isolate peptides that bind to the α_M I-domain, the principal ligand binding site of the integrin. Only one peptide motif, D/E-/D/E-G/L-W, was obtained with this approach in spite of the known ligand binding promiscuity of the I-domain. Interestingly, such negatively charged sequences are present in many known β_2 integrin ligands and also in the catalytic domain of matrix metalloproteinases (MMPs). We show that purified β_2 integrins bind to proMMP-2 and proMMP-9 gelatinases and that the negatively charged sequence of the MMP catalytic domain is an active β_2 integrin-binding site. Furthermore, a synthetic DDGW-containing phage display peptide inhibited the β_2 integrin's ability to bind progelatinases, but did not inhibit the binding of cell adhesion-mediating substrates such as intercellular adhesion molecule-1 (ICAM-1), fibrinogen, or an LLG-containing peptide. Immunoprecipitation and cell surface labelling demonstrated complexes of proMMP-9 with both the $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins in leukocytes, and proMMP-9 colocalized with $\alpha_M\beta_2$ in cell surface protrusions. The DDGW peptide and the gelatinase specific inhibitor peptide CTTHWGFTLC (CTT) blocked β_2 integrin-dependent leukocyte migration in a transwell assay. These results suggest that leukocytes may move in a progelatinase-β_2 integrin complex dependent manner.

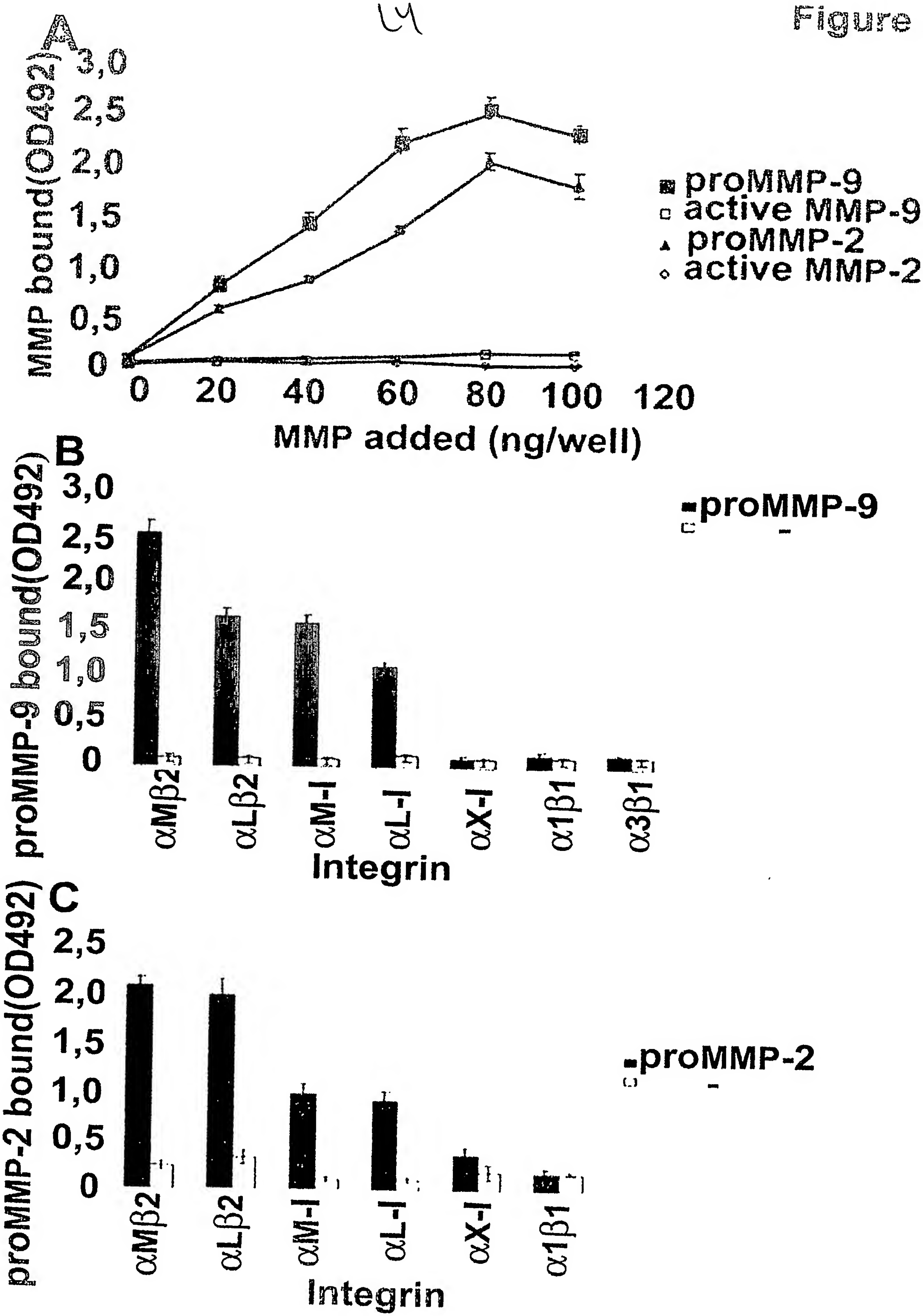
L4

Figure 1



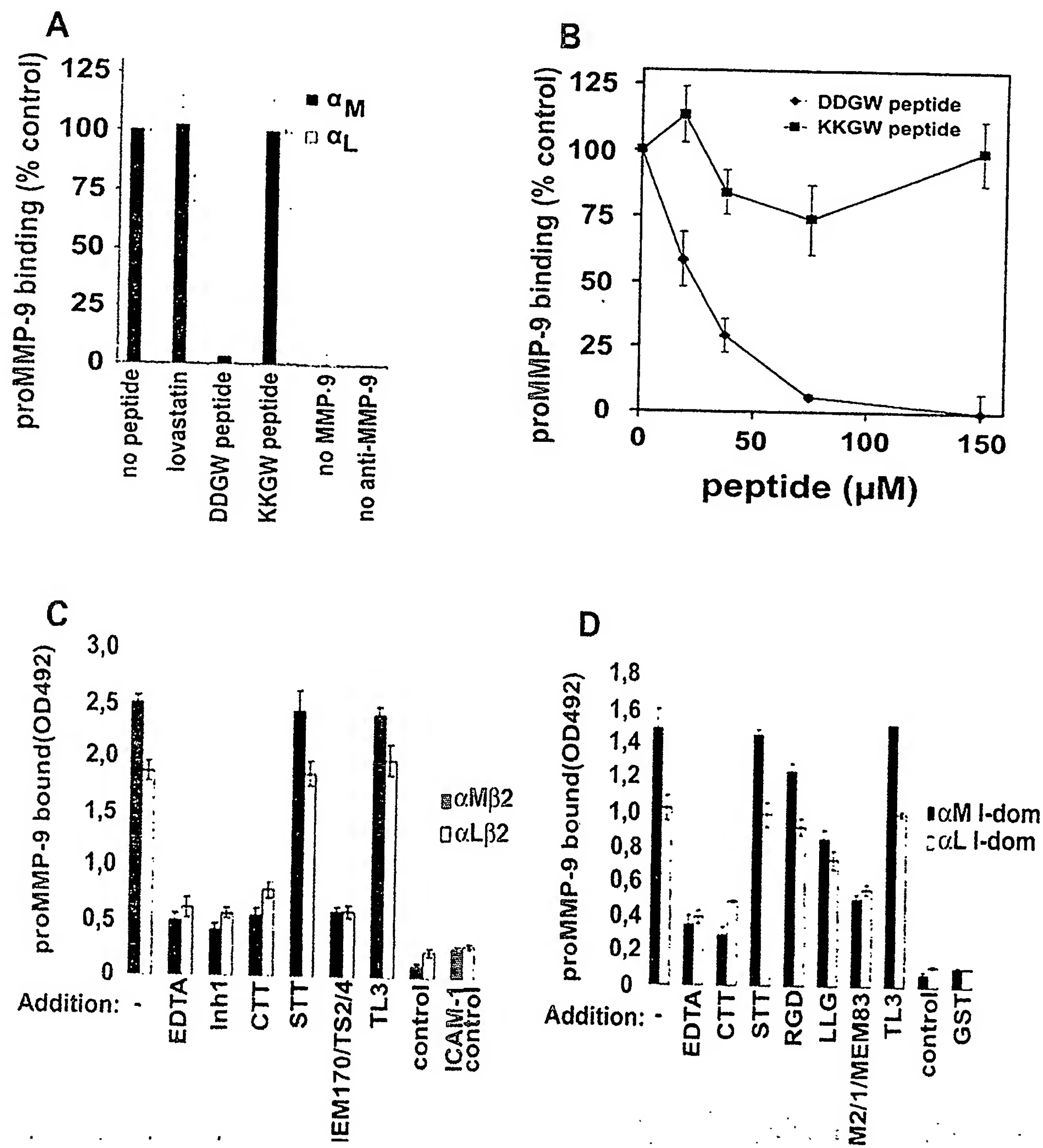
A
Phage sequences
B
Phage binding (Abs 492 nm)
2,000
1,500
1,000
0,500
0,000
No peptide
DDGW peptide
LLG-C4 peptide
DDGW phage
Control phage
C
Phage binding (Abs 492 nm)
α_L - I domain
α_M - I domain
α_X - I domain
GST
DDGW phage
Control phage
D
Pro
Cat
Pex
Peptide 13.
E
DDGW peptide
I→A
L→A
W1→A
M→A
D1→A
D2→A
G→A
W2→A
CILWMDDGWCGAAG
ADGACILWMDDGWC
CILWMDDGWC
ADGACDDGWCGAAG
ADGACDWCGGAAG
ADGACEDGCGAAG
ADGACEDGWCGAAG
ADGACDDELWCGAAG
no peptide
0 500 1000 1500 2000
I domain binding (OD/mm2)

Figure 22


A
MMP bound(OD492)
3,0
2,5
2,0
1,5
1,0
0,5
0
0
20
40
60
80
100
120
MMP added (ng/well)
proMMP-9
active MMP-9
proMMP-2
active MMP-2
B
proMMP-9 bound(OD492)
3,0
2,5
2,0
1,5
1,0
0,5
0
proMMP-9
αMβ2
αLβ2
αM-I
αL-I
αX-I
α1β1
α3β1
Integrin
C
proMMP-2 bound(OD492)
2,5
2,0
1,5
1,0
0,5
0
proMMP-2
αMβ2
αLβ2
αM-I
αL-I
αX-I
α1β1
Integrin

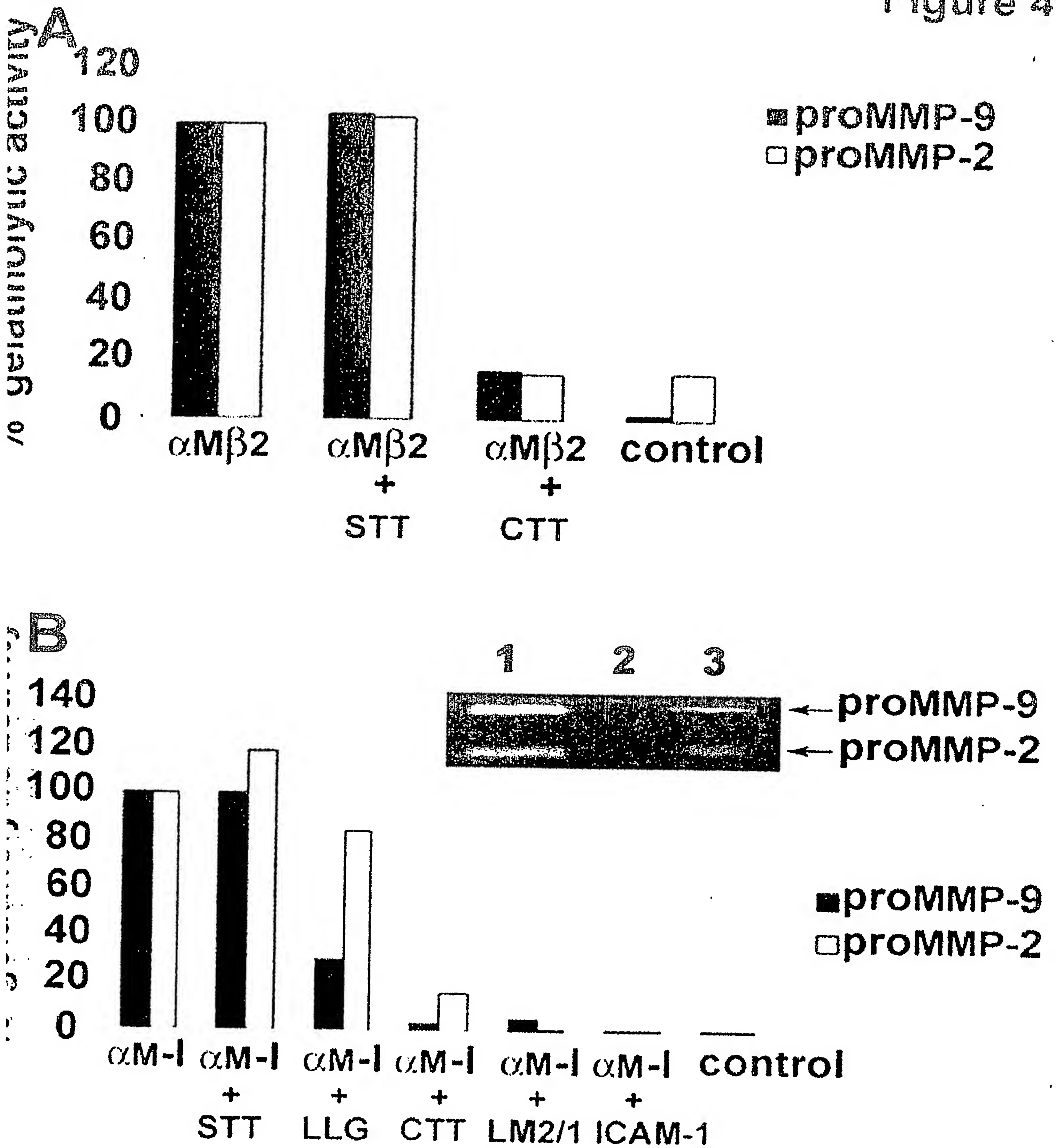
3

Figure 3


A
proMMP-9 binding (% control)
125
100
75
50
25
0
α_M
α_L
no peptide
lovastatin
DDGW peptide
KKGW peptide
no MMP-9
no anti-MMP-9
B
proMMP-9 binding (% control)
DDGW peptide
KKGW peptide
0
50
100
150
peptide (μM)
C
proMMP-9 bound(OD492)
3,0
2,5
2,0
1,5
1,0
0,5
0
αMβ2
αLβ2
Addition: -
EDTA
Inh1
CTT
STT
IEM170/TS2/4
TL3
control
ICAM-1
control
D
proMMP-9 bound(OD492)
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0
αM I-dom
αL I-dom
Addition: -
EDTA
CTT
STT
RGD
LLG
M2/1/MEM83
TL3
control
GST

44 7

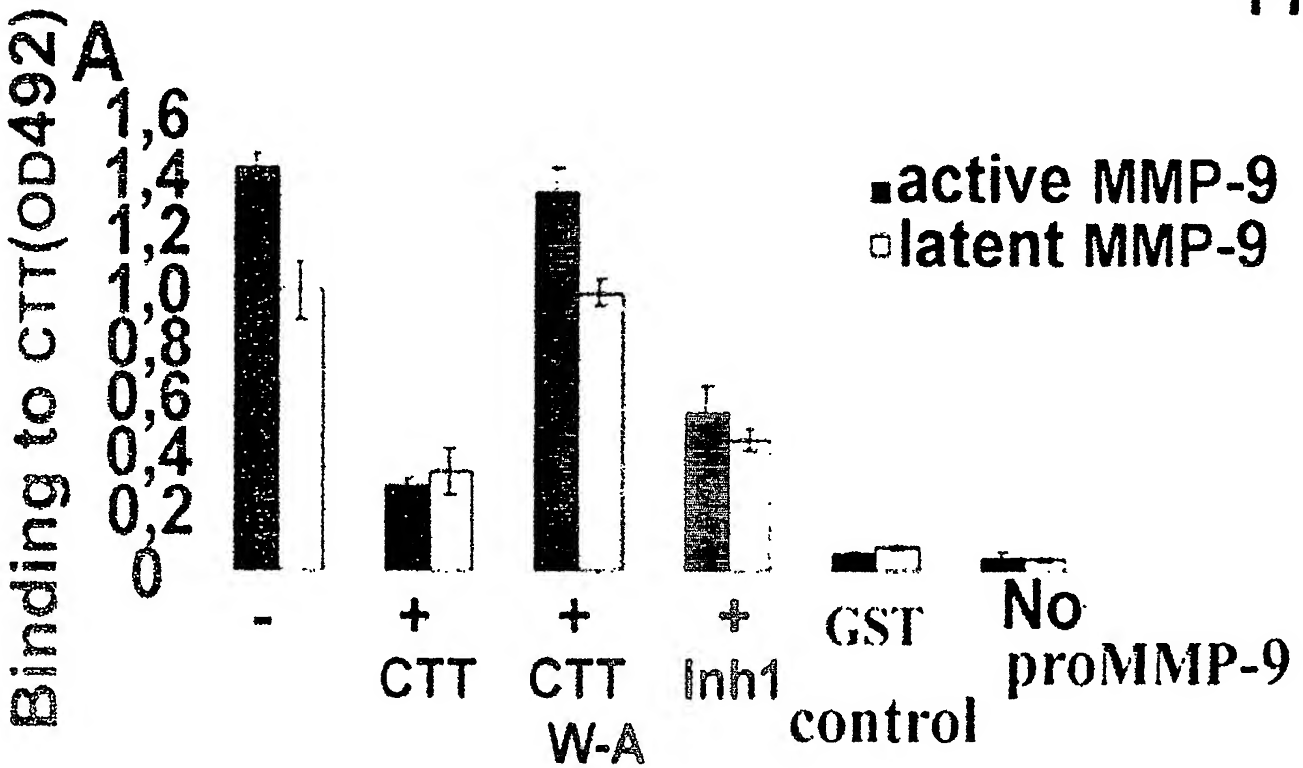
Figure 4



A
% gelatinolytic activity
120
100
80
60
40
20
0
proMMP-9
proMMP-2
αMβ2
αMβ2 + STT
αMβ2 + CTT
control
B
140
120
100
80
60
40
20
0
1 2 3
proMMP-9
proMMP-2
proMMP-9
proMMP-2
αM-I
αM-I + STT
αM-I + LLG
αM-I + CTT
αM-I + LM2/1
αM-I + ICAM-1
control

Figure 5

A


Binding to CTT(OD492)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0
active MMP-9
latent MMP-9
-
+ CTT
+ CTT W-A
+ Inh1
GST control
No proMMP-9

B

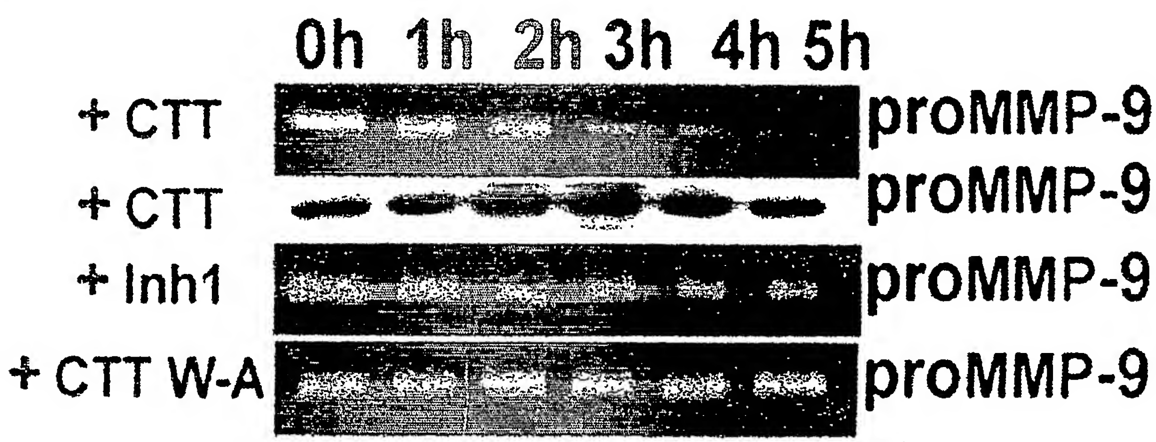
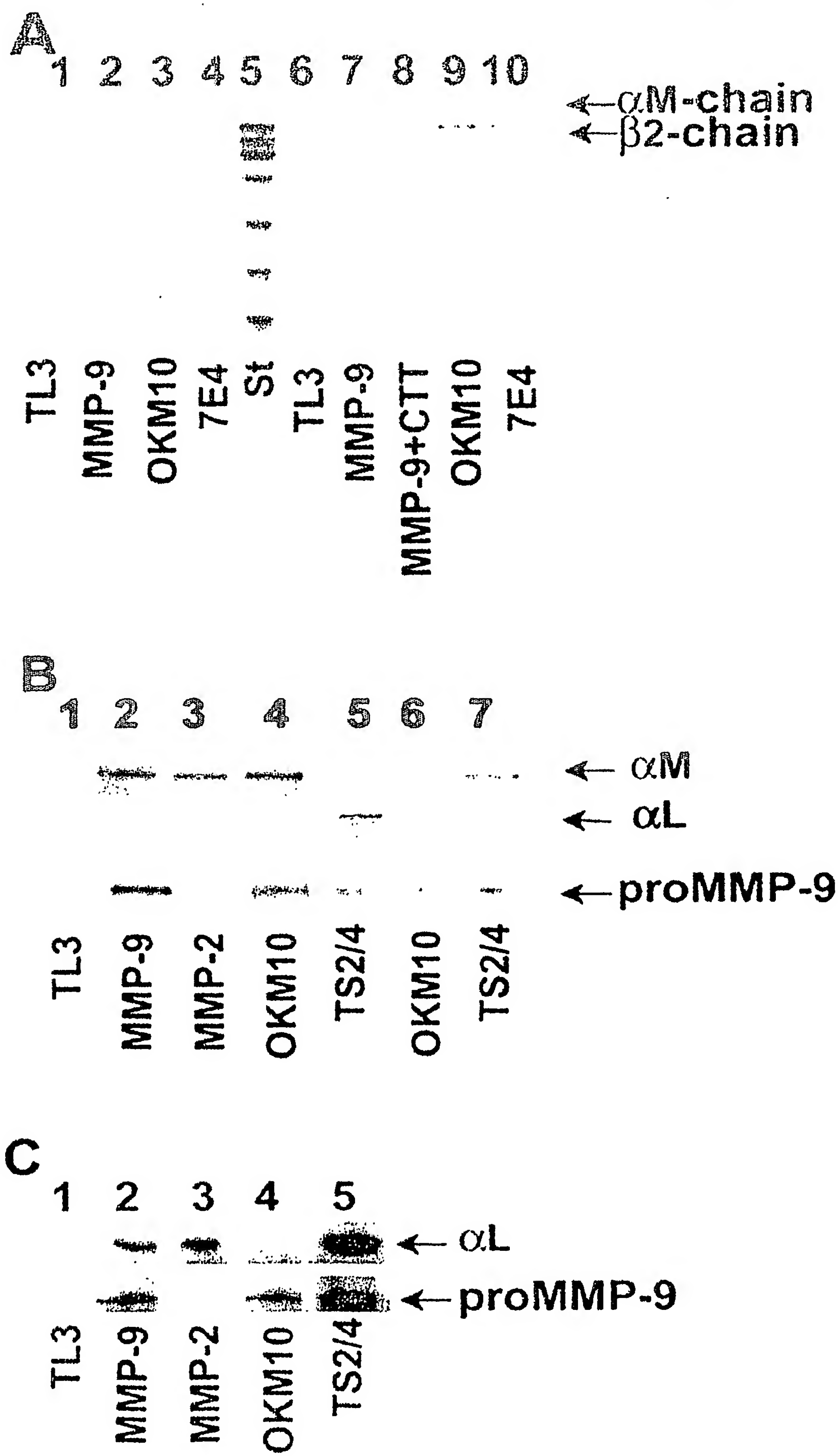

0h 1h 2h 3h 4h 5h
+ CTT
proMMP-9
+ CTT
proMMP-9
+ Inh1
proMMP-9
+ CTT W-A
proMMP-9

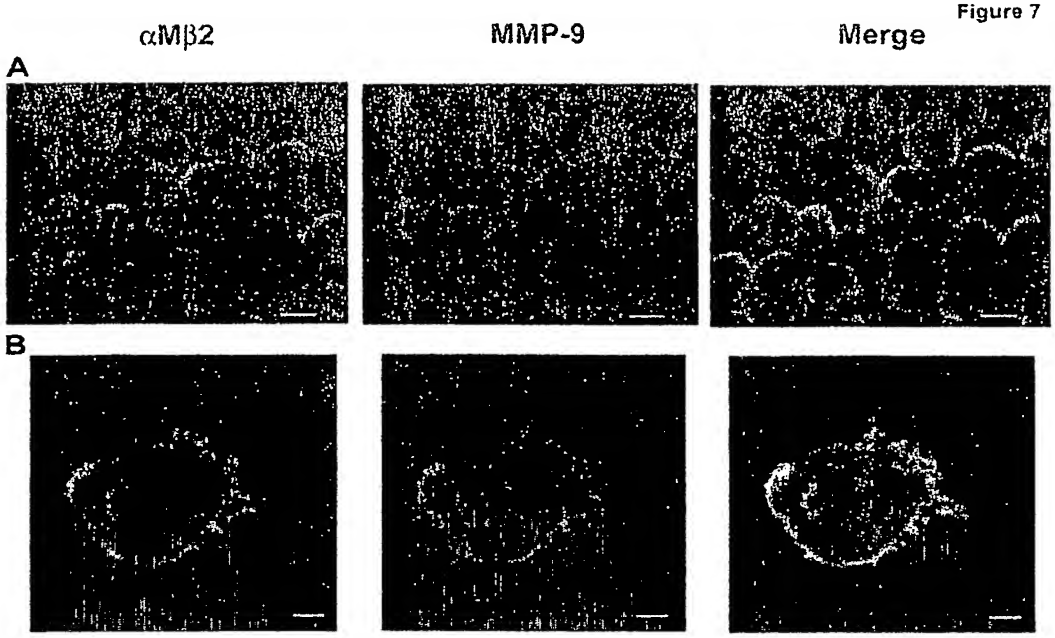
Figure 6


A
1 2 3 4 5 6 7 8 9 10
αM-chain
β2-chain
TL3
MMP-9
OKM10
7E4
St
TL3
MMP-9
MMP-9+CTT
OKM10
7E4
B
1 2 3 4 5 6 7
αM
αL
proMMP-9
TL3
MMP-9
MMP-2
OKM10
TS2/4
OKM10
TS2/4
C
1 2 3 4 5
αL
proMMP-9
TL3
MMP-9
MMP-2
OKM10
TS2/4

L4

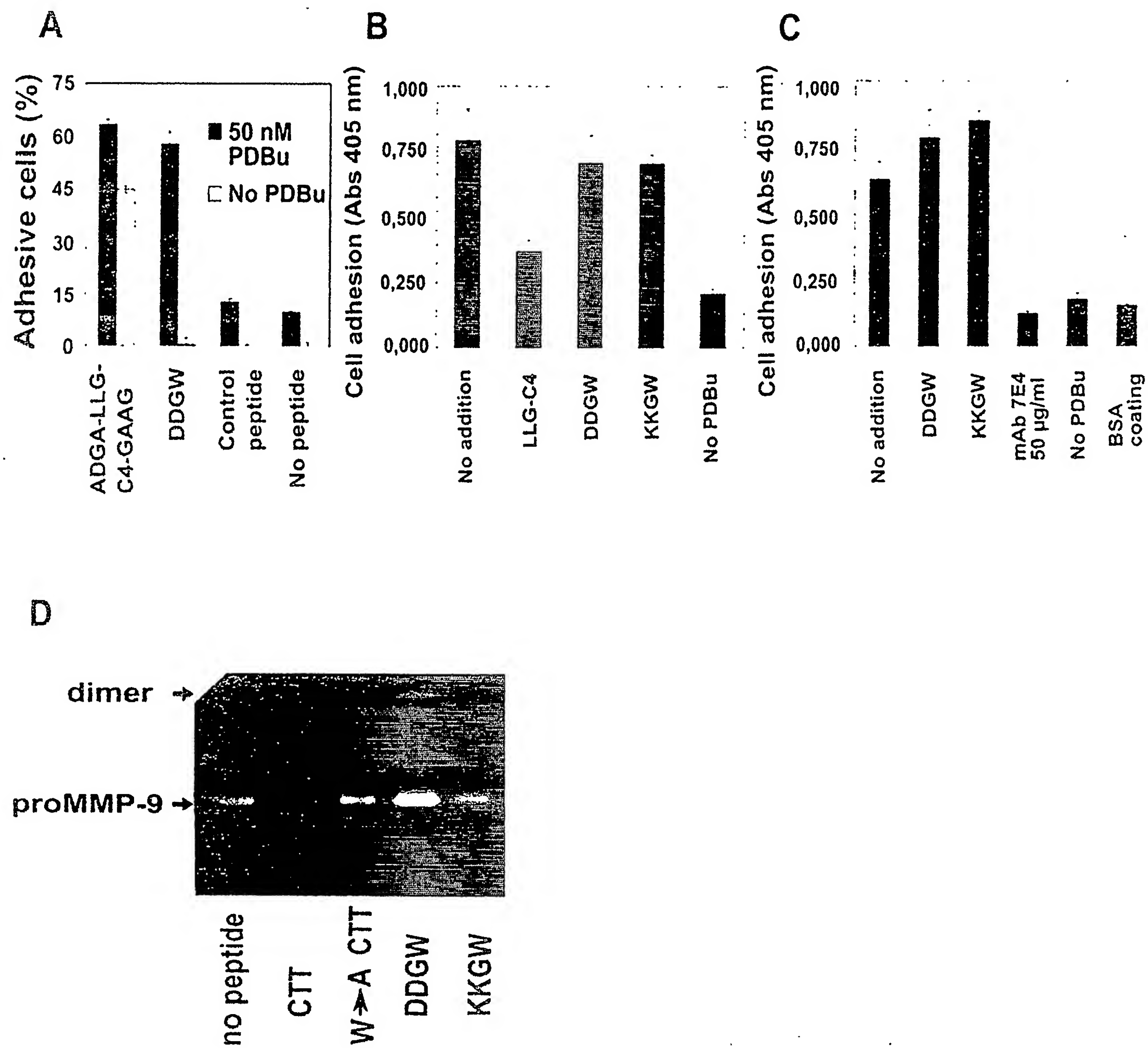
7

Figure 7



8

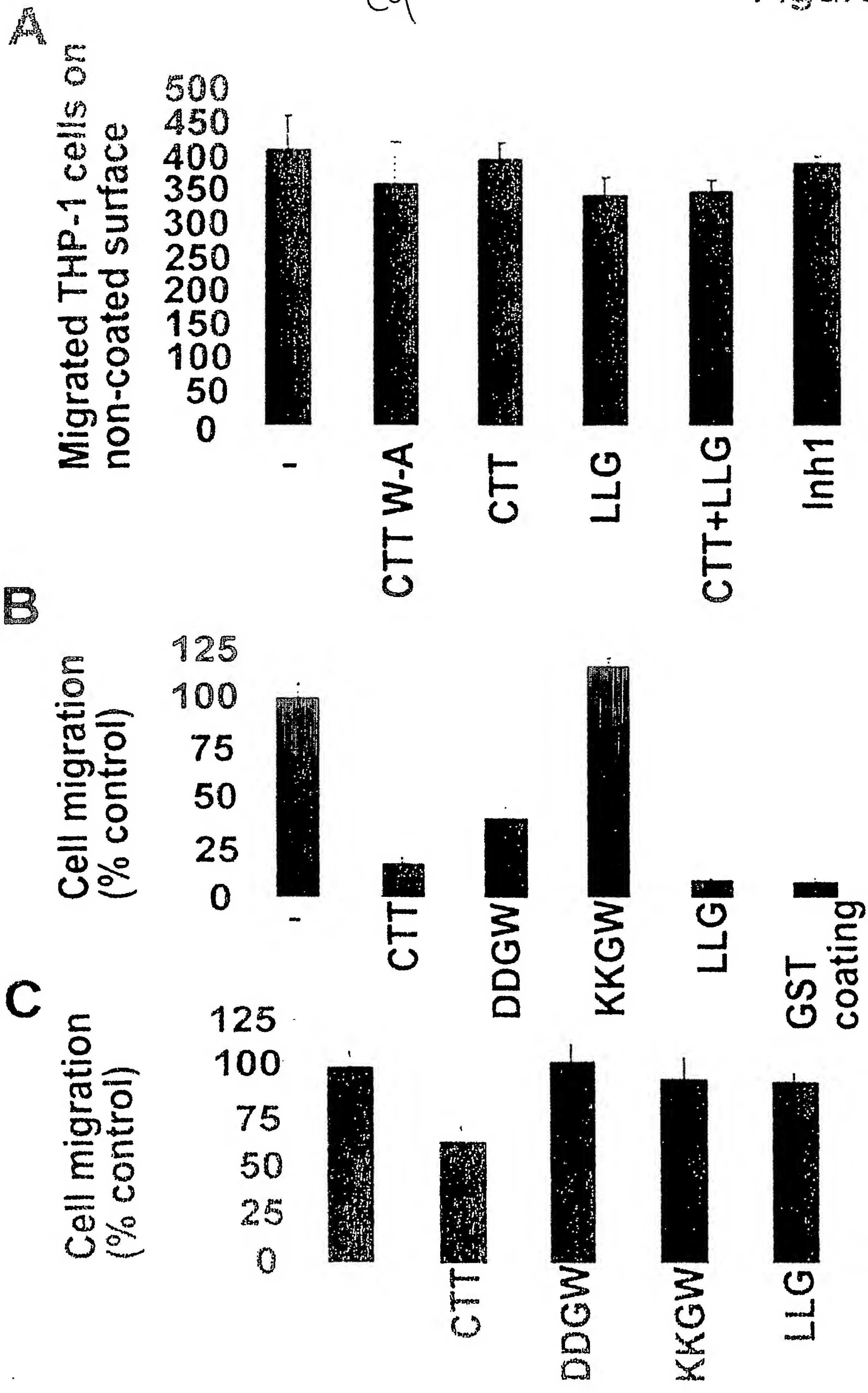
Figure 8


A
Adhesive cells (%)
75
60
45
30
15
0
50 nM PDBu
No PDBu
ADGA-LLG-C4-GAAG
DDGW
Control peptide
No peptide
B
Cell adhesion (Abs 405 nm)
1,000
0,750
0,500
0,250
0,000
No addition
LLG-C4
DDGW
KKGW
No PDBu
C
Cell adhesion (Abs 405 nm)
1,000
0,750
0,500
0,250
0,000
No addition
DDGW
KKGW
mAb 7E4 50 µg/ml
No PDBu
BSA coating
D
dimer
proMMP-9
no peptide
CTT
W→A CTT
DDGW
KKGW

L4

24

Figure 9 9


A
Migrated THP-1 cells on
non-coated surface
500
450
400
350
300
250
200
150
100
50
0
-
CTT W-A
CTT
LLG
CTT+LLG
Inh1
B
Cell migration
(% control)
125
100
75
50
25
0
-
CTT
DDGW
KKGW
LLG
GST
coating
C
Cell migration
(% control)
125
100
75
50
25
0
CTT
DDGW
KKGW
LLG